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***Campylobacter subantarcticus* sp. nov., isolated from birds in the sub-Antarctic region**

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Short title: *Campylobacter subantarcticus* sp. nov.

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41 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of LMG 24377^T, LMG 24374,
42 LMG 24375 and LMG 24378 are AM933371, AM933372, AM933373 and AM933374, respectively. The
43 GenBank/EMBL/DDBJ accession numbers for *hsp60* gene sequences of LMG 24377^T and LMG 24374 are
44 AM933375 and AM933376, respectively.

Abstract

Six Gram-negative, spiral-shaped, micro-aerobic isolates were obtained during a sampling from wild birds in the sub-Antarctic region. Based on initial observations, these isolates were classified as '*Campylobacter lari*-like'. Further characterization was performed by a polyphasic approach, including whole-cell protein and amplified fragment length polymorphism (AFLP) analysis, 16S rRNA and *hsp60* gene sequencing, biochemical analysis, and DNA-DNA hybridizations.

Here, we present comprehensive phylogenetic, genomic and phenotypic evidence that these *C. lari*-like isolates represent a novel species within the genus *Campylobacter*, for which the name *Campylobacter subantarcticus* sp. nov. is proposed. The type strain is R-3023^T (=CCUG 38513^T=LMG 24377^T).

The genus *Campylobacter* (Sebald & Veron, 1963) presently comprises 20 validly named species, and 8 subspecies, with species found in both man and a wide range of domestic and wild animals and birds. Species most often associated with captive or free-living wild birds, either asymptomatic or with disease symptoms, include *Campylobacter lari* subsp. *lari* and *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, and urease positive thermophilic *Campylobacter* (UPTC) *lari* isolates (Waldenstrom *et al.*, 2002; Waldenstrom *et al.*, 2007). The more recently named species *Campylobacter canadensis* has exclusively been isolated from captive whooping cranes (Inglis *et al.*, 2007). The presence of zoonotic species in wild birds may provide a reservoir for human-pathogenic species, either through direct contact or through contamination of the environment.

During a sampling of wild birds and fur seals at Bird Island (54° 00' S, 38° 02' W) in the South Georgian archipelago in 1996, a collection of *Campylobacter* isolates was obtained. Several of these isolates were initially designated *C. lari*-like, based on biochemical similarities. Six of these isolates were included in the present polyphasic taxonomic study: three were isolated from grey headed albatrosses (*Diomedea chrysostoma*), two from black browed albatrosses (*D. melanophris*) and one from a gentoo penguin (*Pygoscelis papua*). No isolates could be obtained from Antarctic fur seals, suggesting that this *Campylobacter* species is restricted to birds. Strains were examined by whole-cell protein SDS-PAGE, AFLP, 16S rRNA and *hsp60* gene sequencing. Phenotypic characteristics were determined, and relevant DNA-DNA hybridisations were performed.

In February / March 1996 fecal swabs were taken from 10 adult female and 40 female Antarctic fur seal pups (*Arctocephalus gazella*), 30 adult gentoo penguins, 50 macaroni penguin chicks (*Eudyptes chrysolophus*), 50 black browed albatross chicks and 50 grey headed albatross chicks. Fecal samples were collected using cotton wool swabs inserted into the rectum or cloaca. Samples were stored in a charcoal transport medium (Transwab, BioDisc, Solna, Sweden) at 5 – 10°C and transported to Sweden within three weeks. Samples were plated on *Campylobacter* selective medium (42.5 g/l Columbia Agar Base,

Becton Dickinson, Cockeysville, Maryland, USA, 5 % citrated horse blood, 10 mg/l Vancomycin, 2500 IE/l Polymyxin B, 5 mg/l Trimetoprim) and incubated for 48 h at 42°C under microaerobic conditions. Colonies showing a Gram-negative seagull-like cell morphology under light microscopy were sub-cultured onto blood agar plates. Samples were stored at -80°C in Trypticase Soy Broth supplemented with 15 % glycerol.

Strains were cultured on Mueller-Hinton agar supplemented with 5% horse blood at 37°C for 48h in microaerobic conditions (approx. 4% O₂, 6.5% CO₂, 6.5% H₂, 83% N₂). DNA was extracted as described by Pitcher *et al.* (1989).

Protein extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Pot *et al.* (1994). For whole-cell protein SDS-PAGE analysis, similarity of the obtained normalized SDS-PAGE patterns was determined by the Pearson product moment correlation coefficient, after which clustering was performed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), using BioNumerics version 4.61 (Applied Maths, Belgium). For numerical analysis a variable dense band region (36.1 – 43.2 kDa) (Vandamme *et al.*, 1990) was excluded to increase species discrimination. The results of the numerical analysis, in combination with visual inspection of the SDS-PAGE patterns, demonstrated that the SDS-PAGE patterns of the novel species were distinct from those of *C. lari*, and all other known *Campylobacter* species (data not shown).

Amplified Fragment Length Polymorphism (AFLP) analysis was performed as described by Debruyne *et al.* (in press). After normalization, the obtained AFLP profiles were included in an in-house AFLP reference database, containing profiles from type and reference strains of all established *Campylobacter* species. The similarity between profiles was determined by the Pearson correlation coefficient, and cluster analysis was performed by UPGMA, using BioNumerics v 4.61. AFLP profiles from the six strains representing the novel species were divergent from those of strains of other *Campylobacter* species, and formed a distinct cluster (Fig. 1).

To support the delineation of the groups defined by the above genomic and proteomic analyses, phenotypic testing was performed. Tests included were evaluation of growth on media containing 1.0% glycine, 0.02% safranin, nalidixic acid (32 mg l⁻¹), cephalothin (32 mg l⁻¹), metrodinazole (4 mg l⁻¹), carbenicillin (32 mg l⁻¹) and 0.1% sodium deoxycholate. Growth on MacConkey agar and unsupplemented nutrient agar (Oxoid no. 2) were also evaluated, as were catalase activity, hippurate hydrolysis, H₂S production on TSI agar, growth at 42°C and α-haemolysis. Methods for biochemical testing were as described previously (On & Holmes, 1991a; 1991b; 1992). Differentiating characteristics are listed in Table 1.

To determine the phylogenetic position of the novel species, 16S rRNA gene sequences of the strains LMG 24374, LMG 24375, LMG 24377^T and LMG 24378 (randomly selected) were determined as described previously (Vandamme *et al.*, 2006). Sequences were assembled using BioNumerics v 5.1. Comparison by the FASTA algorithm to the EMBL sequence database revealed that the nearest phylogenetic neighbours were *C. lari* subsp. *concheus*, *C. lari* subsp. *lari*, *C. jejuni*, *C. coli*, *C. insulaenigrae* and *C. peloridis*, all with similarity levels exceeding 97%. Strains LMG 24375, LMG 24377^T and LMG 24378 had identical 16S rRNA gene sequences (100% sequence similarity), while LMG 24374 was slightly more divergent (99.5%). Sequences were aligned using the ClustalX software package (Thompson *et al.*, 1997), and clustering was performed by the neighbor-joining method (Saitou & Nei, 1987) using BioNumerics v 5.1. Unknown bases were discarded for the analysis. Bootstrap values were determined using 500 replicates (Fig. 2). Polymorphisms within the 16S rRNA gene were inadequate to distinguish among the novel taxon and *C. lari* subsp. *concheus*, with interspecies sequence similarities (99.4-99.9%) being equal to or exceeding intraspecies sequence similarities (99.5-100%). To improve species discrimination, partial *hsp60* gene sequences of LMG 24374 and LMG 24377^T were determined as described before (Debruyne *et al.*, in press). Kärenlampi *et al.* (2004) demonstrated that phylogeny based on the *hsp60* gene sequence, coding for the 60 kDa heat shock protein, was similar to that of the 16S rRNA gene. However, *hsp60* was found to provide a better resolution for *Campylobacter*

species, with lower interspecies sequence similarities and high intraspecies sequence similarities. Pairwise comparison of *hsp60* gene sequences from the novel taxon and from *C. lari* subsp. *concheus* demonstrated a clear separation between intraspecies (100%) and interspecies (93.3-93.9%) sequence similarities, making species discrimination feasible (Fig. 3).

For the determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M (NH₄)H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ -phage DNA (Sigma) was used as the calibration reference. The DNA G+C contents of the strain LMG 24377^T was 30%, which falls within the range reported for genus *Campylobacter*, i.e. 29-47%.

DNA-DNA hybridisations were performed between strain LMG 24377^T and type strains of its closest relatives, i.e. *C. lari* subsp. *lari*, *C. lari* subsp. *concheus*, *C. peloridis*, *C. jejuni* subsp. *jejuni*, *C. coli* and *C. insulaenigrae*. DNA was extracted from 0.25–0.5 g (wet wt) cells as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C. Reciprocal experiments were performed for every pair of strains and standard deviation values ranged from 0.7-7.5. DNA–DNA hybridisation values between strain LMG 24377^T and the type strain of *C. lari* subsp. *lari* (LMG 8846^T), *C. lari* subsp. *concheus* (LMG 21009^T), *C. peloridis* (LMG 23910^T), *C. jejuni* subsp. *jejuni* (LMG 8841^T), *C. coli* (LMG 6440^T), and *C. insulaenigrae* (LMG 22716^T) were 57, 55, 38, 21, 16 and 41%, respectively. All these values are well below the threshold of 70% for species delineation (Stackebrandt & Goebel, 1994).

The present study demonstrates that the six bird isolates represent a novel species within the genus *Campylobacter* which can be distinguished from other *Campylobacter* species by

whole cell protein electrophoresis, AFLP fingerprinting, *hsp60* gene sequence analysis and biochemical characteristics. Below we formally propose to classify these strains as *Campylobacter subantarcticus* sp. nov., with LMG 24377^T (=CCUG 38513^T) as the type strain.

Description of *Campylobacter subantarcticus* sp. nov.

Campylobacter subantarcticus [sub.ant.arc'ti.cus N.L. masc. adj. *subantarcticus*], pertaining to the sub-Antarctic region, from where the organism was isolated.

Cells are slightly curved, Gram negative rods. Colonies are colourless, round, entire, convex, 1-1.5 mm in diameter after culture on 5% blood agar for 72h under microaerobic conditions. Oxidase and catalase positive, strains do not hydrolyse hippurate, and no production of H₂S on TSI agar. Growth at 42°C under micro-aerobic conditions. Growth on media containing 32 mg ml⁻¹ nalidixic acid, and most strains grow on media containing 1% glycine. Most strains do not grow on media containing 4 mg ml⁻¹ metrodinazole or on MacConkey agar. No growth observed on unsupplemented nutrient agar, and on media containing 0.02% safranin, 0.1% sodium deoxycholate, 32 mg ml⁻¹ cephalothin or 32 mg ml⁻¹ carbenicillin. Alpha-haemolysis observed on 5% blood agar

Pathogenicity unknown. Strains have been recovered from wild birds in the sub-Antarctic region. The type strain is R-3023^T (=LMG 24377^T=CCUG 38513^T), which was isolated from a grey headed albatross in 1996.

Acknowledgements

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189 **Figure legends:**

190 Figure 1: Dendrogram representing the AFLP fingerprints of six strains representing the
191 novel species *C. subantarcticus* sp. nov. and selected *Campylobacter* reference strains.
192 Similarity was determined by the Pearson product moment correlation coefficient and
193 clustering was performed by UPGMA.

194

195 Figure 2: Phylogenetic tree based on 16S rRNA gene sequences constructed by the
196 neighbor-joining method. Bootstrap values (%) are indicated at the nodes.

197

198 Figure 3: Neighbor-joining tree based on partial *hsp60* gene sequences. All sequences are
199 555 bp in length, with the exception of the sequence for *C. cuniculorum*, which is 489 bp in
200 length. Bootstrap values (%) are indicated at the nodes.

201

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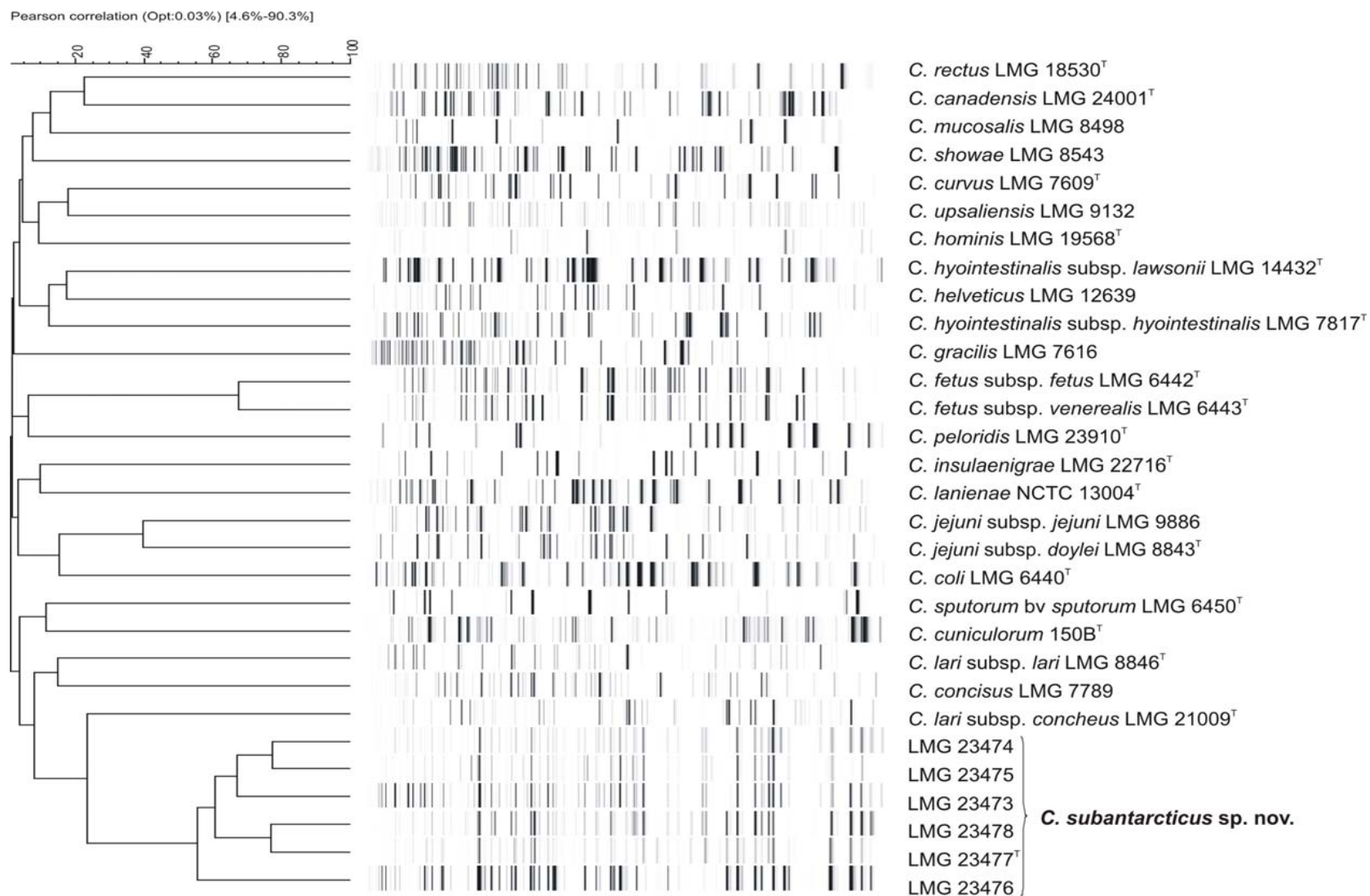
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263 Table 1: Differentiating phenotypic characteristics. 1, *C. subantarcticus* sp. nov. (n=6); 2, *C. canadensis*; 3, *C. coli*; 4, *Campylobacter concisus*; 5,
264 *Campylobacter cuniculorum*; 6, *Campylobacter curvus*; 7, *Campylobacter fetus* subsp. *fetus*; 8, *C. fetus* subsp. *venerealis*; 9, *Campylobacter*
265 *gracilis*; 10, *Campylobacter helveticus*; 11, *Campylobacter hyointestinalis*; 12, *Campylobacter hominis*; 13, *C. insulaenigrae*; 14, *C. jejuni*; 15,
266 *Campylobacter lanienae*; 16, *C. lari* subsp. *concheus*; 17, *C. lari* subsp. *lari*; 18, *Campylobacter mucosalis*; 19, *C. peloridis*; 20, *Campylobacter*
267 *rectus*; 21, *Campylobacter showae*; 22, *Campylobacter sputorum*; 23, *Campylobacter upsaliensis*. +: all strains positive; -: all strains negative; (+):
268 80-94% strains positive; (-): 5-33% strains positive; V: 35-67% positive. Additional data for reference species were taken from Inglis *et al.* (2007),
269 Lawson *et al.* (2001), On *et al.* (1996) and Zanoni *et al.* (in press).

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Catalase	+	V	+	-	+	-	+	(+)	(-)	-	+	-	+	(+)	+	+	+	-	+	(-)	+	V	+
Hippurate hydrolysis	-	-	-	-	-	(-)	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H ₂ S production (TSI)	-	V	-	(-)	-	(-)	-	-	-	-	-	(+)	-	-	-	ND	-	+	ND	-	V	+	-
Growth at 42°C	+	+	+	(+)	(+)	V	(+)	-	(-)	+	+	V	-	V	+	+	+	+	+	(-)	V	(+)	+
Alpha-haemolysis	+	-	(-)	(-)	+	(-)	-	(-)	-	+	V	-	+	(+)	+	ND	+	(-)	ND	+	+	+	+
MacConkey agar	(-)	-	V	-	-	(+)	(+)	V	(+)	-	V	-	-	(-)	+	+	(+)	(+)	+	-	+	V	-
Nutrient agar	-	-	+	(-)	+	+	+	(+)	+	(+)	+	+	V	+	-	+	+	+	+	(-)	V	(+)	+
Glycine (1%)	(+)	V	(+)	(-)	-	+	+	(-)	+	V	V	+	-	V	-	+	+	V	+	+	V	+	+
Safranin (0.02%)	-	ND	+	(-)	ND	+	+	(+)	+	-	+	-	-	V	-	-	+	+	-	-	-	(+)	+

Sodium deoxycholate (0.1%)	-	ND	+	(-)	ND	(+)	+	(+)	(+)	(-)	V	-	+	V	-	V	+	-	V	-	-	V	V
Nalidixic acid (32 mg L ⁻¹)	+	V	-	(+)	V	+	+	V	V	-	+	(+)	+	-	+	-	(+)	(+)	(+)	(+)	-	(+)	-
Cephalothin (32 mg L ⁻¹)	-	-	+	-	(+)	-	-	-	-	-	(-)	-	+	V	+	+	+	V	(-)	-	-	-	(-)
Metrodinazole (4 mg L ⁻¹)	(-)	ND	(+)	(-)	ND	-	(+)	V	-	V	V	-	+	V	+	+	+	(+)	+	-	+	(-)	(+)
Carbenicillin (32 mg L ⁻¹)	-	ND	(+)	-	ND	-	-	-	-	V	-	-	+	V	+	+	+	-	-	-	-	-	-



1%

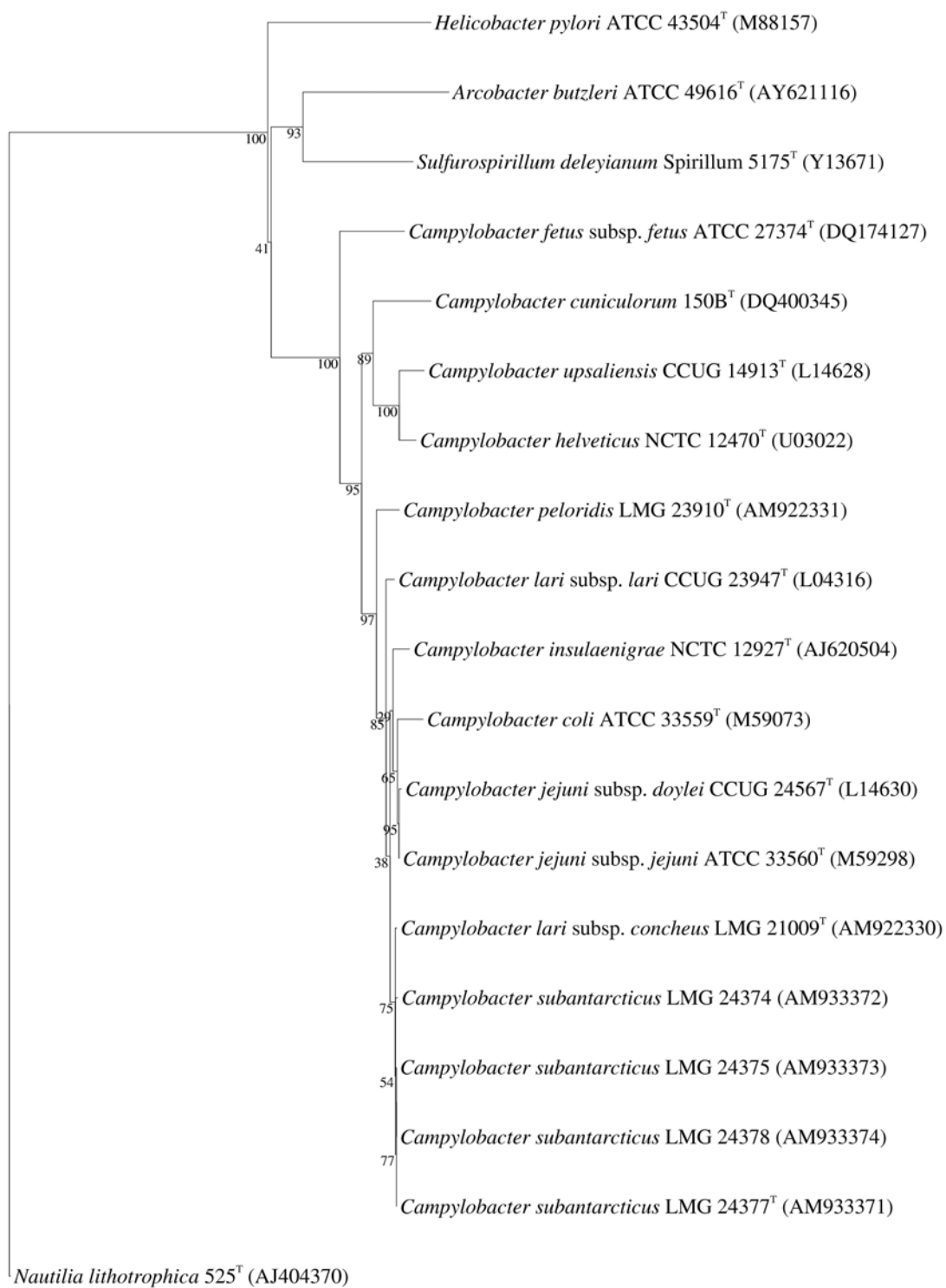


Fig 2

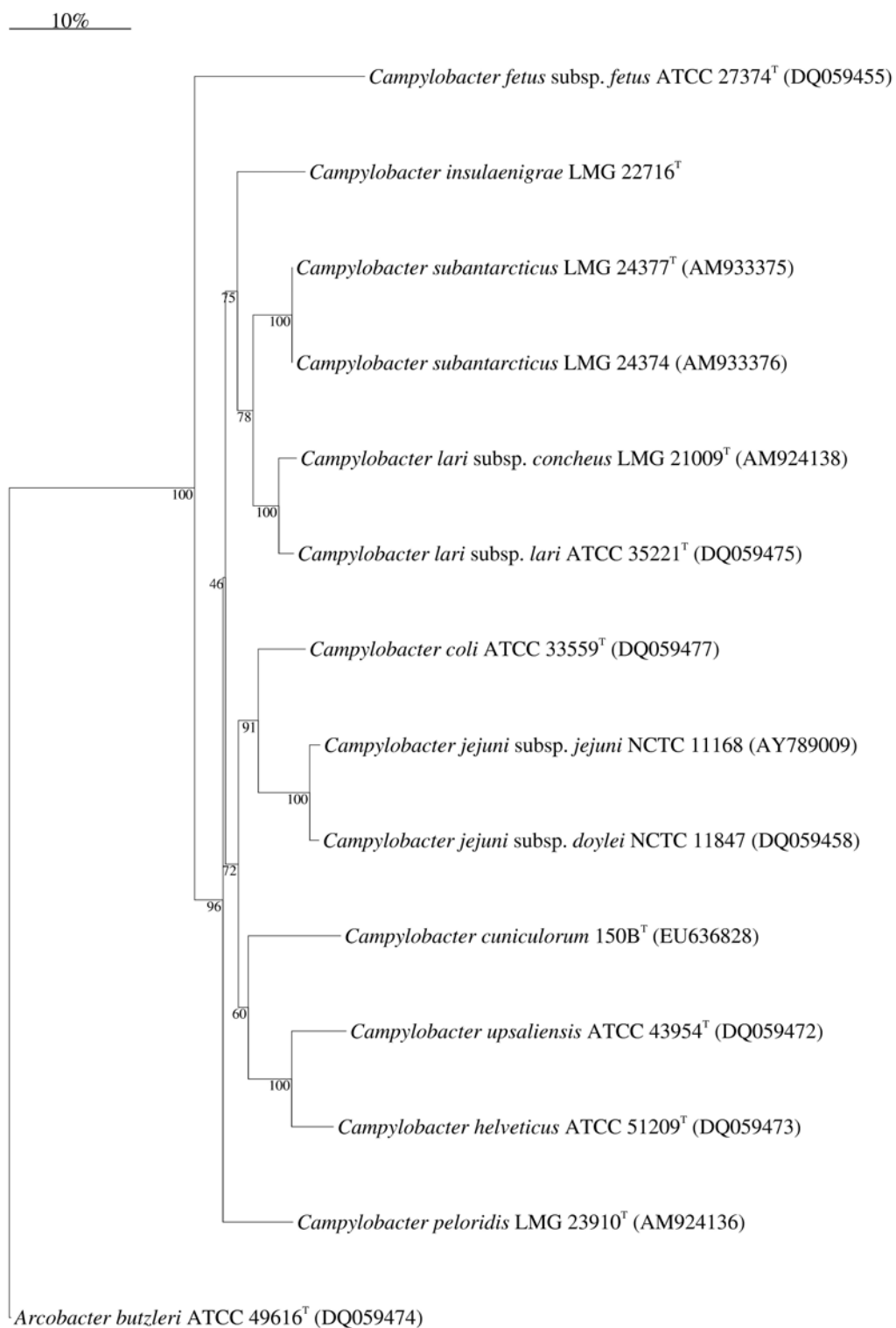


Fig 3